

Experimental model for the study of the effects of platelet-rich plasma on the early phases of muscle healing

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Background. There is abundant evidence suggesting that growth factors may play a key role in the healing process, especially in the early stages of inflammation. Despite the reported clinical successes with the use of growth factors there is still a lack of knowledge on the biological mechanism underlying the activity of platelet-rich plasma during the process of muscle healing. The aim of this study was to analyse the early effects of platelet-rich plasma in an easily reproducible animal model.

Materials and methods. Wistar male adult rats (n=102) were used in this study. The muscle lesion was created with a scalpel in the flexor sublimis muscles. Platelet-rich plasma was applied immediately after surgery. Treated, untreated and contralateral muscles were analysed by morphological evaluation and western blot assay.

Results. Leucocyte infiltration was significantly greater in muscles treated with platelet-rich plasma than in both untreated and contralateral muscles. The latter showed greater leucocyte infiltration when compared to the untreated muscles. Platelet-rich plasma treatment also modified the cellular composition of the leucocyte infiltration leading to increased expression of CD3, CD8, CD19 and CD68 and to decreased CD4 antigen expression in both platelet-rich plasma treated and contralateral muscles. Blood vessel density and blood vessel diameters were not statistically significantly different between the three groups analysed.

Discussion. The results of this study showed that treatment with platelet-rich plasma magnified the physiological early inflammatory response following a muscle injury, modifying the pattern of cellular recruitment. Local platelet-rich plasma treatment may exert a direct or, more plausibly, indirect systemic effect on healing processes, at least in the earliest inflammatory phase.

Keywords: muscle, healing, growth factors, inflammation, repair process.

Introduction

Musculoskeletal injuries are the most common cause of severe, chronic pain and physical disability and account for the majority of all sport-related injuries¹⁻³. Regardless of the mechanism of the injury, the healing process progresses through a constant series of overlapping phases resulting in the restoration of anatomic continuity and function⁴⁻⁶. These phases are controlled by complex and dynamic molecular mechanisms involving local and systemic factors interacting with many different cell types recruited to the injured site from the surrounding tissues and/or circulation. There is abundant evidence suggesting that growth factors may play a key role in the healing process, especially in the early stages of inflammation⁷⁻¹². These observations constituted the basis for the use of platelet-rich plasma (PRP) in several circumstances, all of them characterised by the need to activate, modulate, accelerate or ameliorate the process of tissue repair. Clinically, it has been

reported that the application of PRP to injured muscles can reduce symptoms and shorten the recovery period, allowing early mobilisation¹³. These observations have been confirmed in animal models using both PRP¹⁴ and platelet-rich fibrin matrix¹⁵. Despite the reported clinical successes and the many studies demonstrating the biological efficacy of isolated growth factors¹⁶⁻¹⁹, there is still a paucity of data on the biological mechanisms underlying the activity of PRP during the muscle healing process. Moreover, the review of the literature reveals a lack of standardisation in both the preparation of PRP and its application^{20,21}. These observations may explain the variability in clinical and experimental results obtained in different studies²². Finally, the impact of the use of growth factors should be considered in the light of anti-doping regulations. Indeed the World Anti-Doping Agency (WADA) included intramuscular injection of PRP among their 2010 list of prohibited substances²³, then allowed its use in the current 2012 list²⁴. This

modification was made despite the suggested systemic effect of locally injected growth factors described by some authors²⁵. The aim of this study was to analyse the early effects of PRP in an easily reproducible animal model, and to verify whether local administration of PRP may exert a systemic effect on contralateral muscles.

Materials and methods

Animals and surgery

Wistar male adult rats (n = 102), weighing approximately 250 g each, were used. Eighteen animals were used for the set up of the experimental model (i.e. blood collection, PRP preparation, muscle lesion and PRP injection in the injured site). Animals were sacrificed 1, 3 and 7 days postoperatively for macroscopic and microscopic assessment of the healing of the lesion. Fifty-four rats were sacrificed in order to investigate the time course of leucocyte infiltration and to study its characteristics at 1, 2, 3, 4, 5 and 6 days after the injury. To evaluate a possible systemic effect, an injury was made to the right flexor muscles of 18 rats and immediately treated with PRP (treated group), 18 animals, used as controls, were subjected to the same muscle injury and left untreated (control group), 18 rats were subjected to muscle injury in both anterior limbs; the injury to the right limb was treated with PRP while the injury on the left remained untreated (contralateral group). Finally 30 rats were used for western blot analysis in order to evaluate the composition of the leucocyte infiltration: 15 animals were sacrificed 2 days after surgery (5 in the control group, 5 in the treated group and 5 in the contralateral group). The remaining 15 animals were sacrificed 5 days after surgery (5 in the control group, 5 in the treated group and 5 in the contralateral group). All animals were kept in a cage in a room with controlled temperature and humidity, with a light/dark cycle of 12/12 hours, and allowed food and water *ad libitum*. Animals were operated under general anaesthesia by intramuscular injection of tiletamine + zolazepam (Zoletil) 3 mg/kg. The surgical procedures were performed with the aid of a surgical microscope (Zeiss OPMI7, Jena, Germany). A longitudinal incision was performed on the right anterior limb (or both anterior limbs) from the elbow region to the wrist in order to access the flexor sublimis muscles of the upper joint of the digits. The muscle was then injured transversely and medially using a scalpel. The wedge-shaped lesion was 3 mm long, 2 mm wide and 3 mm deep. Immediately after the incision, the injury sites of the treated animals were filled with PRP. Although the type of surgery adopted ensured very limited post-operative discomfort and virtually no signs of animal suffering, all rats were treated with a pain killer (Finadyne administered intramuscularly at a dose of 2.5 mg/kg/12 h). The decision to give all animals the analgesic, independently

of the presence of signs of suffering, was made with the intent of obtaining the same conditions in the different experimental settings since the use of anti-inflammatory drugs may affect both the healing process and the early inflammatory response²⁶. All procedures were approved by the local Animal Care Committee, supervised by a veterinarian, and performed in accordance with the Local Ethical Committee's rulings and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Blood collection and preparation of platelet-rich plasma

Blood was collected by intracardiac puncture of the anaesthetised rats immediately before the surgical injury to the muscle. Briefly, a needle (21G) was inserted at the base of the sternum at a 20° angle just lateral of the midline. The intracardiac blood (3-3.5 mL) was slowly withdrawn into a syringe containing 1 mL of 3.8% sodium citrate. Blood was then transferred into sterile tubes containing sodium citrate and underwent a first centrifugation at 220 g for 15 minutes. To objectively determine the number of platelets and investigate the presence of other blood cells, a complete blood count was performed using a cell counter ADVIA 201 (Bayer, Leverkusen, Germany) on a small amount of the plasma layer obtained after the first centrifugation (the white blood cell content was always very low: $(0.01 \pm 0.082) \times 10^3/\text{mm}^3$, P-PRP subtype²⁷). A second centrifugation at 1,270 g for 5 minutes allowed the platelets to fall to the bottom of the tube. The pellet was re-suspended in 100 µL of plasma (the platelet concentration was always at least four times greater than the initial value). This platelet enriched preparation was activated with 20 µL of 10% calcium chloride (Braun, Melsungen, Germany, 1,000 IE/mL CaCl_2 -2SG) at 37 °C and, after jellification, immediately inserted using tweezers into the injured muscle of the same animals from which the blood had been drawn. The wound was then sutured and washed with saline solution.

Light microscopy

Muscles were fixed in 4% paraformaldehyde for 2-4 hours and then washed in phosphate-buffered saline. Samples were dehydrated in an ascending series of graded alcohol, cleared in xylene and embedded in paraffin. Series of longitudinal and transverse sections of the muscles were obtained using a microtome RM2135 (Leica Microsystems, Wetzlar, Germany). The following histological stains were used: haematoxylin and eosin, Masson's trichrome and May Grunwald-Giemsa.

Morphoquantitative stereological analysis

Morphoquantitative stereological analysis was performed on serial sections stained with haematoxylin

and eosin. A microscope equipped with a digital camera DM4000B DFC320 and software for processing images IM50 (Leica Microsystems) was used²⁸. For the stereological quantification of the volume of the infiltrate, a design-based systematic-random sampling scheme and the Cavalieri method were adopted^{29,30}. In addition, the unbiased counting frame method³¹ was used to estimate the total number of leucocytes infiltrating the muscle as well as the blood vessel density in the area of infiltration.

Immunohistochemistry and confocal laser microscopy

Sections were permeabilised, blocked [0.3% triton X-100, 10% normal goat serum/0.1% NaN₃, for 1 h] and processed for immunohistochemical studies. Sections were incubated overnight in primary antibody and visualised using a solution containing the appropriate secondary antibody. The primary antibody used was MyoD (BD Pharmingen, Franklin Lakes, New Jersey). Goat anti-mouse IgG Alexa-Fluor-568-conjugated and Fluorescein goat anti-mouse IgG1 (Life Technologies, Paisley, UK) were used as secondary antibodies. Finally, samples were mounted with a Dako fluorescent mounting medium and analysed using a LSM 510 confocal laser microscopy system (Zeiss, Jena, Germany) equipped with an inverted Axiovert 100M microscope.

Western blot assay

Samples of muscle tissue (200-300 mg) were homogenised and lysed in RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulphate) supplemented with Halt™ Protease Inhibitor Cocktail. Whole cell lysates were obtained by subsequent lysis with politron. Protein concentrations were determined using a Bradford Protein Assay Kit with bovine serum albumin as a standard. Protein extracts (100 µg) were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a Protran nitrocellulose membrane. This membrane was incubated with CD3 (abcam, Cambridge, UK), CD4 (Abbiotec, San Diego, California, USA), CD8 (Abbiotec), CD19 (Abbiotec), CD68 (Abbiotec) and MyoD (BD Pharmingen) at 4 °C overnight. The membrane was washed with TBST buffer (20 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, and 0.1% Tween-20), incubated with a secondary goat anti-rabbit (Santa Cruz Biotechnology, Dallas, Texas, USA) and goat anti-mouse (Santa Cruz Biotechnology) for 2 h at room temperature. The immuno-reacting bands were visualized by exposing the membranes to Kodak X-OMAT films. Densitometry and quantification were performed using the ImageJ software.

Statistical analysis

Database management and all statistical analyses were performed using the Statistica 6 for Windows

software package (Statsoft Inc., Tulsa, OK, USA). Differences between means were assessed with a two-tailed Student's t test fixing the level of statistical significance to $P \leq 0.05$. Correlations between quantitative variables were calculated by Pearson's r test. The null hypothesis of no correlation ($\rho = 0$) was tested using the Fischer transformation. Results were considered statistically significant when P was < 0.05 . The comparisons among the groups examined (control vs treated vs contralateral) were evaluated by analysis of variance (ANOVA) and a post hoc Bonferroni's test for multiple comparisons.

Results

Experimental set up of the muscle injury model

Macroscopic observation allowed us to establish the timing of muscle recovery following the lesion, which was particularly early in the selected experimental model. Indeed, 3 days after the lesion the wound appeared to be almost completely closed (the edges of the wound were almost completely adherent) and at 7 days the muscle appeared very similar to a non-injured muscle. Since the physiological regenerative process was extremely rapid and at 7 days after its creation the lesion was no longer macroscopically detectable, we decided to limit the timing of the analytical determinations to the first 6 days after the muscle injury even if, microscopically, a very small leucocyte infiltration was still present 30 days after the injury (Figure 1).

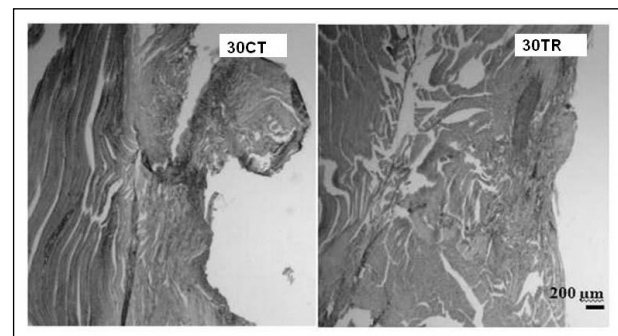


Figure 1 - Optical microscopy images of control (CT) and treated (TR) muscles at 30 days after surgery. Magnification, 2.5X; haematoxylin and eosin staining.

Light microscopy

Figures 2 and 3 show representative microscopic pictures taken at different postoperative time points from the control, contralateral and treated groups. The leucocyte infiltration around the lesion was clear and appeared to increase over time until day 5 after the surgical injury. Seven days after the injury, scar tissue completely closed the wound and the leucocyte infiltration progressively spread away from the lesion site.

Volume of the leucocyte infiltration

The longitudinal sections enabled us to quantify the total volume of the leucocyte infiltration around the site of the lesion (Figure 4). The volume of the leucocyte infiltration, in the group treated with PRP was significantly greater than that in both the control and the contralateral groups.

Significant differences also emerged when analysing the infiltration volume of the contralateral samples. The leucocyte infiltration in the contralateral group was greater than that in control group. Thus suggesting intermediate values between the treated and the untreated samples when considering the immediate inflammatory response.

Number of cells

When considering the total number of white blood cells infiltrating the injury site (Figure 5), the stereological analysis confirmed that the number of leucocytes was greater in the treated samples than in the other two groups. It is interesting that the number of cells infiltrating the muscles of the contralateral

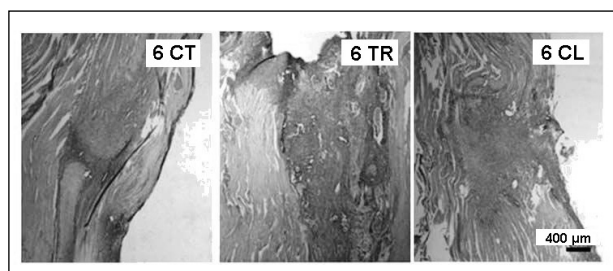


Figure 2 - Optical microscopy images of control (CT), treated (TR) and contralateral (CL) muscles at 6 days after surgery. Magnification, 2.5X; haematoxylin and eosin staining.

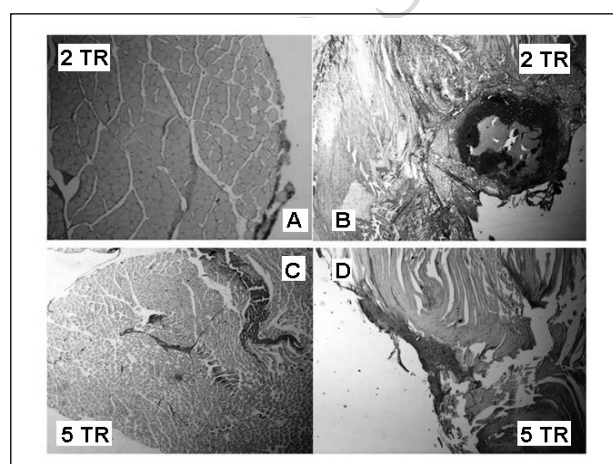


Figure 3 - Optical microscopy images of treated muscles at 2 and 5 days. Longitudinal (B, D) and transverse (A, C) sections at magnification 2.5X (B, C, D) and 10X (A); haematoxylin and eosin staining.

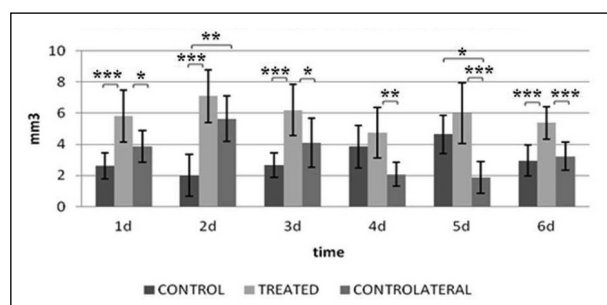


Figure 4 - Leucocyte infiltration volume. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$.

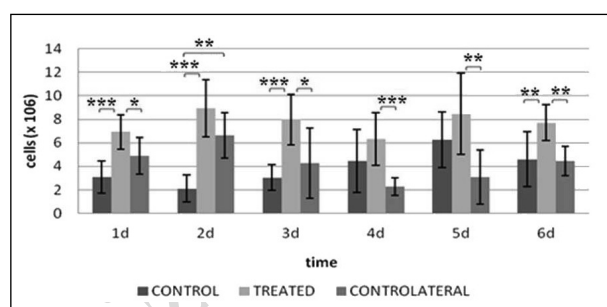


Figure 5 - Number of cells. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$.

group showed a distinct behaviour when compared to the control group. Indeed, cells were more abundant in the first 3 days and then this increase reversed during the following days.

Composition of the leucocyte infiltrate

When considering the composition of the leucocyte infiltration, there were slightly more lymphocytes and monocytes in the muscles treated with PRP than in the control and contralateral groups.

Blood vessel density and diameter

With regard to the number of blood vessels in the area of the lesion, the microscopic analysis at 10X magnification showed a not statistically significant increase in the number of vessels in the samples treated with PRP when compared to the untreated muscles and to contralateral ones (Figure 6). Similarly, the analysis of the diameter of blood vessels showed that there were no statistically significant differences among the three groups considered.

Protein assay

PRP treatment modified the cellular composition of the leucocyte infiltration leading to increased expression of CD3, CD8, CD19 and CD68 in the treated and contralateral muscles when compared to the control ones (Figure 7). In contrast, CD4 expression was decreased in both the PRP-treated and contralateral muscles in

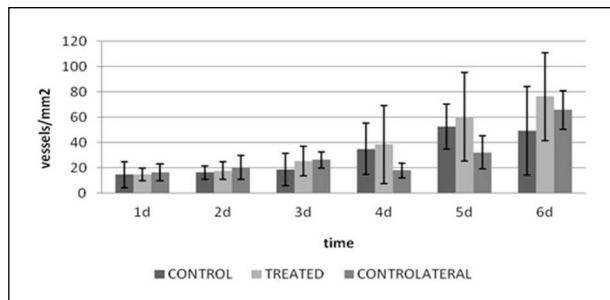


Figure 6 - Vessel density.

comparison to the expression in the control samples (Figure 7). Again, values in contralateral muscles were intermediate between those in the treated and the untreated samples. Indeed, CD3, CD8, CD19 and CD68 expression was higher in contralateral muscles than in untreated muscles but lower than that in PRP-treated muscles. Similarly, CD4 expression in contralateral muscles was lower than that in control samples but higher than that in PRP-treated samples.

Immunohistochemistry and western blot analysis showed higher expression of MyoD antigen 2 and 5 days after the surgically induced lesion in the treated group when compared to the others two groups but this difference was statistically significant only at 2 and 5 days after surgery in the following comparisons: treated vs control ($P \leq 0.01$); treated vs contralateral ($P \leq 0.05$) and treated vs control ($P \leq 0.05$) (Table I, Figure 8).

Discussion

The results of the present study showed that PRP treatment magnifies the physiological early inflammatory response following a muscle injury, with a concomitant modification of the pattern of cellular recruitment. Moreover, it has been clearly demonstrated that PRP treatment may exert, directly or, more plausibly, indirectly, systemic effects.

Physiologically, during the early inflammatory phase, it has been demonstrated that blood vessels are naturally torn, and consequently, blood-borne inflammatory cells gain direct access to the site of injury^{32,33}. The beginning of the inflammatory response is amplified by the release of various substances by satellite cells and necrotic regions of the injured muscle fibres, which serve as chemoattractants in order to

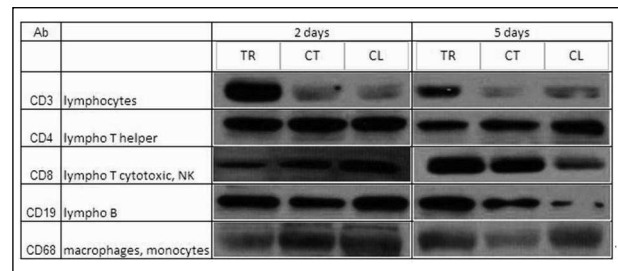


Figure 7 - Analysis of cell types and subtypes in the leucocyte infiltrate at 2 and 5 days in the three groups analysed (TR: treated, CT: control and CL: contralateral).

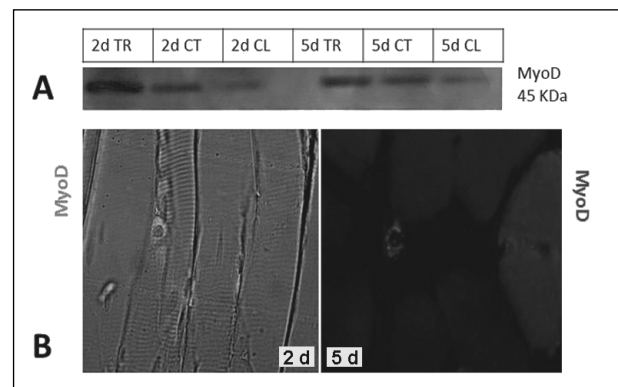


Figure 8 - A. MyoD muscle protein expression at 2 and 5 days in the three groups analysed (TR: treated, CT: control and CL: contralateral). B. Immunohistochemistry analysis (MyoD+) in treated muscle at 2 (left) and 5 days (right) after surgery.

facilitate the transition of pro-inflammatory cells from the blood vessels at the site of injury^{32,34,35}. Within the injured muscle, fibroblasts and macrophages are then activated, producing additional chemoattractants. In addition, most tissues contain growth factors stored in an inactive form to be used in case of immediate need³⁶. It has been clearly shown in numerous studies that during the acute phase following a muscle injury, polymorphonuclear leucocytes are the most abundant cells present in the area of the lesion^{5,37-40}, but that within the first days these cells are replaced by monocytes. In accordance with the basic principles of inflammation, monocytes are then actively involved in proteolysis and phagocytosis of necrotic material^{5,32,41,42}. This process is highly specific and is aimed to preserve the cylinders of the basal lamina surrounding the necrotic

Table I - Analysis of MyoD protein expression at 2 and 5 days in the three study groups.

	2d TR vs CT	2d CL vs CT	2d TR vs CL	5d TR vs CT	5d CL vs CT	5d TR vs CL
MyoD	**	ns	*	ns	*	*
	2d TR	2d CT	2d CL	5d TR	5d CT	5d CL
MyoD	33,02±2,86	13,2±1,70	16,72±2,39	15,49±5,57	13,66±1,42	8,82±1,66

Legend TR: treated, CT: control and CL: contralateral. *: $P \leq 0.05$; **: $P \leq 0.01$.

parts of the muscle fibres to be used as scaffolds in which the satellite cells can begin the formation of new muscle fibres^{5,43,44}. Despite a huge amount of data regarding the effects of different isolated growth factors on muscle healing processes^{18,19,45}, little is known about the mechanism by which plasma preparations with high concentrations of growth factors can modulate the inflammatory response²⁵. Moreover, a review of the literature reveals a lack of standardisation with regards to both the preparation and the administration of PRP which may explain the inconsistent clinical and experimental results obtained in different studies⁴⁶⁻⁴⁹. A second important limitation derives from the fact that the vast majority of human studies have been performed on small samples usually with very small numbers of evaluable controls^{7,45,50}. Most of the studies do not, therefore, have sufficient statistical power to support the adoption of the tested approach in clinical routine⁵⁰. The results of the present study, in which an easily reproducible animal model was used, demonstrated that early PRP treatment significantly amplifies the physiological inflammatory response, as suggested by some authors, and modifies the pattern of inflammatory cell recruitment when compared to a physiological response to a muscle injury⁵¹. These observations contrast with those of Gigante *et al.* who, in their study performed on Wistar rats treated with platelet-rich fibrin matrix, did not observe any effect on inflammation¹⁵. This discrepancy may be partially due to the different preparations used, once again highlighting the need for standardisation of PRP preparation and administration before its adoption in clinical routine.

The absence of significant differences in both blood vessel density and diameter among the three groups considered in this study suggest that PRP is not able to stimulating neo-angiogenesis in muscle directly. Again, these findings contrast with those of other studies performed with different PRP preparations¹⁵ or carried out on different tissues⁵². It is possible that different tissues may have different biological responses to PRP administration and that different PRP preparations may induce different biological pathways. These issues certainly need further assessment.

The higher expression of MyoD in treated samples observed on days 2 and 5 after surgery suggests that PRP may generate a myogenic response during the early phases of the regenerative process. This observation confirms the findings of other studies in animal models suggesting that muscle regeneration occurs in the early phase of healing and that PRP can stimulate myogenesis^{14,15,19}.

Taken together, these observations may have some immediate clinical translations. Indeed, the demonstrated modulation of the inflammatory response may explain the pain reduction usually observed

after PRP administration and account for the early mobilisation of treated patients^{16,53,54}.

To assess a potential systemic effect of PRP, "contralateral" muscles were analysed. These samples showed an intermediate response between the control group and the treated one. It does, therefore, seem that PRP administration may exert a systemic effect when healing processes are concerned, at least during the very first stages of inflammation. Considering the short half-life of growth factors, in the order of minutes⁵⁵, it is difficult to consider that growth factors released locally by platelets may act on contralateral injured muscle. This observation agrees with the findings of recent studies in which the systemic levels of some growth factors, such as vascular endothelial growth factor and epidermal growth factor, were modified within 24 hours after PRP administration^{25,56}. Further studies are needed to clarify this issue, with particular regard to anti-doping purposes^{23,24}.

In conclusion, the results of the present study confirmed that PRP is a promising treatment for muscle injuries but that there are still unresolved issues. Further experimental studies are needed to fully understand the local and systemic mechanisms of action before using PRP in routine clinical practice. The easy reproducibility of the model used in this study has allowed us to create a solid foundation on which future studies will be carried out in order to deepen the understanding of the molecular dynamics of the inflammatory process modulated by PRP administration as well as the early myogenic response.

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The Authors declare no conflicts of interest.

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